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Evidence of a Heparin-Binding Protein in the Plasma of the Slipper Oyster, *Crassostrea iredalei*

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Abstract: Recognition proteins play an important role in the immunodefense system of invertebrates. The haemolymph of the Slipper oyster, *Crassostrea iredalei*, contains a heparin-binding protein with a molecular weight of 35 kDa as determined by SDS-PAGE analysis. The protein was purified using column chromatography. This binding protein possesses a serine protease activity thus it is capable of activating the prophenoloxidase-activating (proPO) system. However, the protein lacks β-1,3-glucanase activity. Using rabbit antiserum against the isolated protein in immunodiffusion and immunoblotting assays it produced a single precipitant and a single band, respectively. However, N-terminal amino acid sequence of BGBP: Threonine-Alanine-Arginine-Asparagine-Glutamic acid-Alanine-Asparagine-Valine was similar to Cavortin (AAT44352) and extracellular superoxide dismutase (AAY60161) from *C. gigas*.

Key words: Heparin-Binding Protein (HBP), *Crassostrea iredalei*, 2D-electrophoresis, proPhenoloxidase-activating (proPO) system, Malaysia

INTRODUCTION

Invertebrates possess an Innate Immune System but lack specialised cells that store an immunologic memory. Hence, they possess several other mechanisms to defend themselves from foreign molecules and one of these defences is the prophenoloxidase-activating (proPO) system (Cerenius and Soderhall, 2004). This system is humoral but possesses no memory however, the reactions are immediate and complete. This system is initiated by recognition which is followed by a proteolytic process through a serine protease cascade. The end product of the system involves melanin which acts by engulfing foreign molecules.

The proPO system begins with a Pattern Recognition Protein (PRP) (Soderhall and Cerenius, 1998). For example, recognition proteins such as β-1,3-Glucan-Binding Protein (BGBP) act by binding to foreign molecules that contain β-1,3-glucan. This binding activity which uses a glycosylation site, promotes a serine protease cascade (Duvic and Soderhall, 1990; Jayaraj *et al*., 2008; Roux *et al*., 2002; Yoshida *et al*., 1986; Zhang *et al*., 2003). Subsequently, a zymogen prophenoloxidase activation enzyme (ppA) is activated and eventually activates proPO to phenoloxidase (PO) (Ashida and Soderhall, 1984; Cerenius and Soderhall, 2004). PO is known to oxidise monophenols to o-diphenols and the further oxidation of o-diphenols to o-quinones and quinones leads to non-enzymatic polymerisation to melanin (Cerenius and Soderhall, 2004; Soderhall and Cerenius, 1998).

However, it is not known whether the proPO system in mollusc bivalves is identical to that of crustaceans as proposed by Soderhall and other researchers (Ashida and Soderhall, 1984; Cerenius *et al*., 2008; Cerenius and Soderhall, 2004; Soderhall and Cerenius, 1998). Bivalves predominantly live underwater and are more exposed to the environment. Several studies have been performed to investigate the proPO systems in the plasma of bivalves such as Sydney rock oysters (*Saccostrea glomerata*) and Pacific oysters (*Crassostrea gigas*) (Hellio *et al*., 2007; Newton *et al*., 2004; Thomas-Guyon *et al*., 2009).

In elucidating the proPO system, significant research has been performed to isolate and characterise their components including BGBP. A first attempt to purify a BGBP was performed from the plasma of the death's head cockroach, *Blaberus craniifer* and the silkworm, *Bombyx mori* (Ochiai and Ashida, 1988; Soderhall *et al*., 1988). BGBP has now been isolated, purified and characterised widely in different phyla. In the bivalve mollusc phylum, Jayaraj *et al*. (2008) were the first to

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isolate and characterise a BGBP from the mussel *Perna viridis*. A BGBP was also identified from *C. gigas* and was present in multiple copies on the cDNA (Itoh *et al*., 2010). In addition, lipopolysaccharide and beta-1,3-Glucan-Binding Protein (*LBGBP*) gene expression have been obtained from the scallop *Chlamys farreri* (Su *et al*., 2004).

Apart from binding to β-1,3-glucan, BGBP also binds to heparin sulphate in a specific manner (Jimenez-Vega *et al*., 2002). The BGBP of the white shrimp, *Penaeus vannamei* is also reported to be a Heparin-Binding Protein (HBP) (Jimenez-Vega *et al*., 2002). However, there is currently no report on the HBP of the slipper oyster, *Crassostrea iredalei* in relation to the proPO system.

This study was conducted to isolate and purify HBP from the slipper oyster, *C. iredalei* and to characterise its biochemical properties as related to the proPO system.

MATERIALS AND METHODS

Animals: Approximately, 50 Slipper oysters, *C. iredalei* with shell lengths ranging from 3.0-8.5 cm were collected from the Setiu Wetland, Terengganu, Malaysia (N5°40′38.6″; E102°43′3.2″). At the sampling time, the temperature and salinity were 30.5°C and 29.5 ppt, respectively.

Plasma preparation: Approximately, 2 mL of haemolymph was withdrawn from the pericardial cavity near the adductor muscle and clarified as previously described (Asokan *et al*., 1997). The haemolymph was centrifuged at 400×g for 10 min (4°C). The supernatant (whole plasma) was used for further analysis.

Column chromatography using a heparin-sepharose column: The whole plasma was dialysed against double distilled water overnight. The dialysed supernatant was centrifuged at 10,000×g for 15 min (4°C) prior to column chromatography analysis.

A heparin-sepharose pre-packed HiTrap™ Heparin HP column (GE Healthcare, Uppsala, Sweden) was prepared, as earlier described (Jimenez-Vega *et al*., 2002). The column was equilibrated with TBS A (50 mM Tris-HCl, 200 mM NaCl, pH 7.5). Whole plasma was injected at a flow rate of 0.5 mL min^{-1}. The column was then washed with TBS A to remove unbound protein. The heparin-bound protein was eluted using step-gradient elution with TBS B (50 mM Tris-HCl, 1 M NaCl, pH 7.5) at a flow rate of 1.5 mL min^{-1}. Fractions with a peak absorbance at 280 nm were collected at 1 mL per fraction (eluted plasma HBP).

Protein concentration determination: The protein concentrations in the whole plasma and eluted plasma HBP were determined using the Bradford (1976) Method with Bovine Serum Albumin (BSA) served as a standard.

Enzymatic assays

Phenoloxidase assay: The PO assay was performed using a microplate format as previously described (Asokan *et al*., 1997). About 50 μL of whole plasma or eluted plasma HBP was incubated with 50 μL laminarin (1 mg mL^{-1}) at 25°C for 5 min. About 50 μL of L-dihydroxyphenylalanine (L-DOPA; Sigma-Aldrich, St. Louis, MO, USA) was added to the mixture which was further incubated at 25°C for 10 min. The formation of dopachrome was read using a spectrophotometer at an absorbance of 480 nm.

A dose response study was also performed using different concentrations of eluted plasma HBP. The sample was serially diluted (2 fold) with TBS A before being incubated with laminarin as described. BSA was used as a negative control while trypsin (TPCK-treated from bovine pancreas; Sigma, St. Louis, MO, USA) was used as a positive control.

Serine protease assay: Serine protease activity was measured using a previously described method (Erlanger *et al*., 1961; Jayaraj *et al*., 2008). About 25 μL of whole plasma or eluted plasma HBP was incubated with 25 μL of laminarin (1 mg mL^{-1}) at 25°C for 10 min. About 25 μL of Nα-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA; Sigma-Aldrich, St. Louis, MO, USA) substrate along with 75 μL TBS A was mixed and the mixture was incubated at 37°C for 30 min. The reaction mixture was terminated by the addition of 50 μL of 50% (v/v) acetic acid prior to measuring the absorbance at 405 nm. BSA was used as a negative control while trypsin (TPCK-treated from bovine pancreas; Sigma, St. Louis, MO, USA) was used as a positive control.

β-1,3-glucanase assay: β-1,3-glucanase activity was measured using a Microplate Method (Dygert *et al*., 1965; Zheng and Wozniak, 1997). The measurement was based on a spectrum reading of neocuproine. About 10 μL of whole plasma or eluted plasma HBP was incubated with 20 μL laminarin at 25°C for 30 min. After the incubation, 100 μL of copper reagent and 100 μL of neocuproine reagent were mixed with the sample. The mixture was then boiled for 10 min prior to the absorbance reading at 450 nm which was performed by microplate reader (Bio Rad, Hercules, CA, USA). For the control, the sample was heated at 95°C for 7 min before being incubated with laminarin.

Protein molecular weight and isoelectric point determination

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was performed as earlier described (Laemmli, 1970) using a 3% stacking gel, 13% resolving gel and a non-continuous buffer system. Samples of either whole plasma or eluted plasma HBP were mixed with sample buffer (0.125 M Tris-HCl, 4% (w/v) SDS, 20% (w/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (w/v) bromophenol blue, pH 6.8; Sigma, St. Louis, MO, USA) at a ratio of 1:1. About 5 μL of sample was run in parallel with the protein molecular weight markers (Invitrogen, Carlsbad, CA, USA) which consisted of myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66.3 kDa), glutamic dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), carbonic anhydrase (31.0 kDa) and trypsin inhibitor (21.5 kDa) for molecular mass determination. The gel was stained using Coomassie Brilliant Blue (Sigma-Aldrich, St. Louis, MO, USA).

2nd dimension of the electrophoresis

1D Electrophoresis, isoelectric Focusing (IEF): IEF was performed using a previously described protocol (Gorg *et al*., 2000). Briefly whole plasma and eluted plasma HBP were mixed with rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT and 0.5% (v/v) IPG buffer, pH 3-10 NL; Amersham, Piscataway, NJ, USA) and later applied to a gel strip IPG pH 3-10 (7 cm). The gel strip was rehydrated for 12 h. The IEF was performed using an IPGphor IEF unit (Amersham Pharmacia, Piscataway, NJ, USA) and programmed as follows: 500 V (30 min), 1000 V (30 min) and 5000 V (1.5 h). The gel strip was then equilibrated with reducing equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (w/v) glycerol, 2% SDS, 1% DTT (w/v) and trace bromophenol blue) for 15 min followed by alkylation equilibration buffer (reducing equilibration buffer with DTT replaced by 1% (w/v) iodoacetamide) prior to 2D electrophoresis, SDS-PAGE.

2D electrophoresis, SDS-PAGE: The gel strip was placed in a well of a 13% polyacrylamide gel layered with 0.5% agarose. Electrophoresis was run at 114 V for 1 h. The gel was stained with Coomassie Brilliant Blue, scanned and analysed using the Software ImageMaster 2D Platinum and Melanie Viewer (GE Healthcare, Uppsala, Sweden).

Antiserum production and immunoassay: Antiserum against the eluted plasma HBP was produced in rabbit. The antiserum titre was further determined using ELISA and tested against HBP by immunodiffusion (Bailey, 1996) and immunoblotting. For immunoblotting, the samples were subjected to SDS-PAGE. After the electrophoresis, the proteins were electrotransferred to PVDF membrane for 1 h at 100 V in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3) using a Mini Trans-Blot System (Bio-Rad, Hercules, CA, USA). After the transfer, the PVDF membrane was blocked in washing buffer (3% (w/v) BSA and 0.02% (v/v) Tween-20, pH 7.2 in PBS) for 2 h washed with PBS before being incubated with antiserum (diluted 1:10,000 in PBS) for another 1 h washed twice with PBS for 15 min, overlaid with donkey anti-rabbit IgG coupled with a horseradish peroxidise conjugate (HRP; Thermo Scientific, Waltham, MA, USA) for 1 h washed twice again for 15 min with PBS and finally assayed with diaminobenzidine tetrahydrochloride dehydrate (0.1 M Tris-HCl. 0.7 mM diaminobenzidine, 0.01% (v/v) H_2O_2, pH 7.6) as has been earlier described (Towbin *et al*., 1979; Yepiz-Plascencia *et al*., 1998).

N-terminal sequencing: N-terminal protein sequencing was done using an ABI Procise Model 491 Edman Micro Sequencer connected with ABI Model 140C PTH Amino Acid Analyzer. The N-terminal sequencing was processed according to a chemical reaction cycle (Edman, 1950). Sequence similarity was done using BLAST network service (http://www.ncbi.gov./Blast) and aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2).

Statistical analyses: Each experiment was reopeated at least 3 times with each experiment run in triplicate. The mean differences between the control and tested value were analysed using a paired-sample Student's t-test. For the dose response study, the relationship between different HBP concentrations and dopachrome release was tested using Pearson's correlation coefficient (r). The probability was $p<0.05$. Statistical analyses were performed using the Software Statistical Package for the Social Sciences (SPSS) 16.0 (IBM, Armonk, NY, USA).

RESULTS AND DISCUSSION

Column chromatography using a heparin-sepharose column: Following a earlier described protocol (Jimenez-Vega *et al*., 2002), researchers obtained one major peak along with a few shoulder peaks (Fig. 1a). Thus, some modifications as previously described (Clairbois *et al*., 1998) were made to obtain a single peak with a good chromatographic resolution. These modifications involved using buffered Tris (50 mM Tris-HCl, pH 7.5) for the equilibration and washing steps while adding a few gradient steps (0.15 M, 0.2 M and 1 M) to the elution step (Fig. 1b). Fractions at the desired peak

were collected, pooled and re-concentrated in TBS A using a Vivaspin concentrator (Sartoriusstedim, Germany). The yield of the collected proteins was 8% (Table 1).

Enzymatic assay: Eluted plasma HBP significantly enhanced the PO activity of the effluent ($p<0.05$) in the presence of laminarin (Fig. 2a). Furthermore, the PO activity increased with an increased concentration of eluted plasma HBP (Fig. 2b; $r = 0.92$, $p<0.05$). Purified HBP displayed a serine protease activity ($p<0.05$, Fig. 2c) but lacked a β-1,3-glucanase activity ($p>0.05$, Table 2).

Protein molecular weight and isoelectric point determination: Compared to whole plasma, eluted plasma HBP demonstrated a single band of 35 kDa when estimated by SDS-PAGE (Fig. 3a). About 100 g each of whole plasma and eluted plasma HBP was further subjected to 2D electrophoresis. The results indicated that the whole plasma and eluted plasma HBP dissociated into 66 and 5 spots, respectively (Fig. 3b and c). The 5

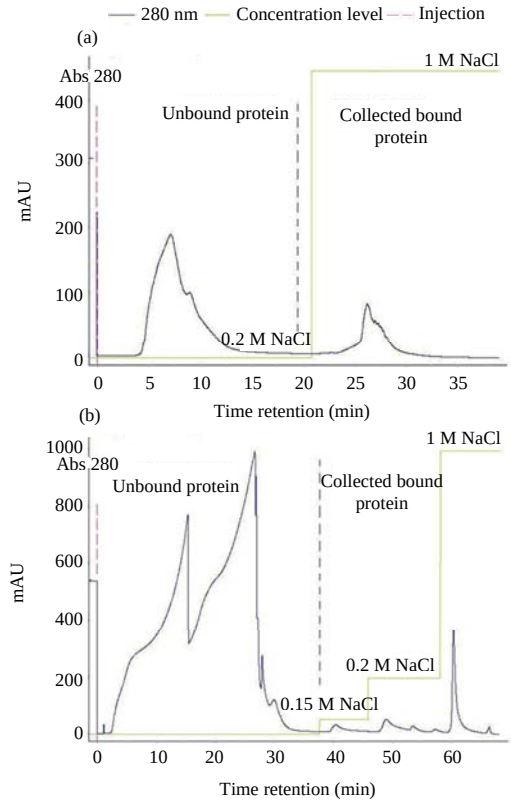

Fig. 1: Affinity chromatography using a HiTrap Heparin column. a) A major peak with a shoulder peak appeared when the elution was performed with TBS containing 1 M NaCl. b) A single peak with a good chromatographic resolution was present after a series of step gradient elutions with NaCl

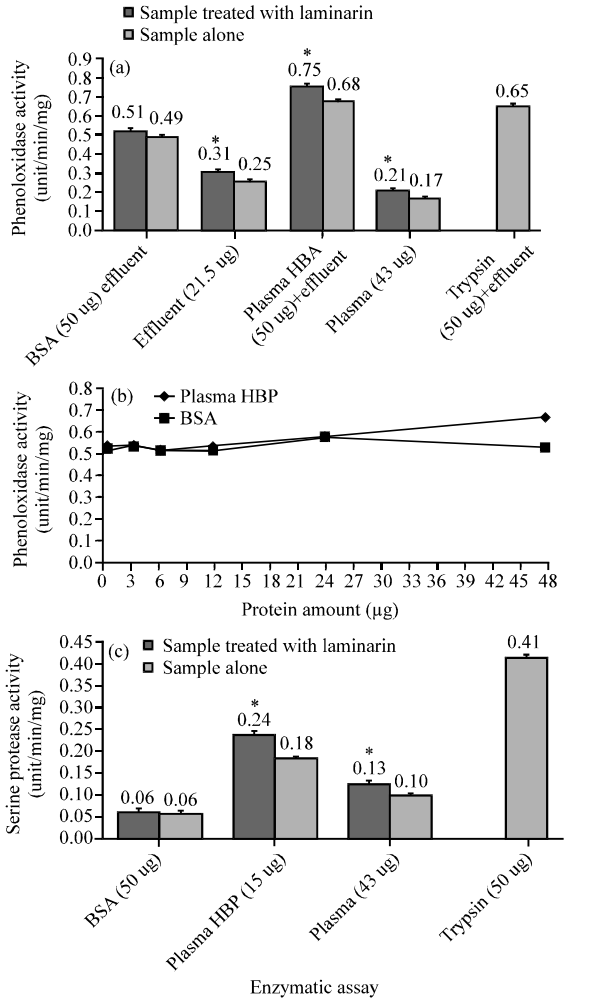

Fig. 2: a) Enhancement of PO activity in plasma and effluent by plasma HBP (50 µg). b) Effect of different concentrations of plasma HBP on the PO activity of the effluent ($r = 0.92$, $n = 18$), compared to BSA ($r = 0.21$, $n = 18$). c) Serine protease activity in the plasma and plasma HBP. *Values for the laminarin-treated sample were significantly different than those for the untreated sample values ($p<0.05$)

Table 1: Summary of the plasma HBP purification using affinity chromatography and a HiTrap Heparin column

Samples	Volume (mL)	Concentration (µg mL^{-1})	Total protein content (mg)	Yield of captured protein% (colected eluent (mg)/injected crude (mg)×100)
Processed plasma (crude)	56.5	440	24.86	100.00
HBP	1.0	2016	2.00	8.04

spots from the eluted plasma HBP were similarly found in whole plasma and found to be collectively distributed into 2 regions, α and β (Fig. 3d). However, these spots displayed a similar molecular mass (37-38 kDa) but different pIs (Table 3).

Antiserum production and immunoassay: As detected using ELISA, the antiserum was obtained at a high titre (1:625,000). The immunodiffusion assay indicated that the antiserum not only detected eluted plasma HBP with a single precipitation but also detected protein in the effluent with multiple precipitations (Fig. 4). Immunoblotting indicated a single band of 35 kDa for both the whole plasma and the eluted plasma HBP detected by the antiserum (Fig. 3a).

N-terminal sequencing: Comparison using BLAST network service showed that this eight amino acid sequence of eluted plasma HBP from *C. iredalei* is similar to Cavortin (AAT44352) and extracellular superoxide dismutase (AAY60161) from *C. gigas* (Fig. 5).

The presence of heparin in invertebrates (including bivalves) has been associated with cell recognition and growth control (Dietrich, 1984; Medeiros *et al*., 2000; Porcionatto *et al*., 1998). It is possible that the existence of a HBP is associated with the osmoregulation process or the immune defence-related process. It has been proven that the HBP found in the white shrimp, *Penaeus vannamei* is BGBP (Jimenez-Vega *et al*., 2002). BGBP has a specific affinity towards heparin (Jimenez-Vega *et al*., 2002). The present study indicates that HBP was present in the *C. iredalei* plasma. In addition to its binding capability through a highly specific interaction, heparin also serves as a cation exchanger (Xiong *et al*., 2008). Heparin contains a sulphate group, making it the most negatively charged sugar. Different proteins have different binding affinities towards heparin. This binding strength is based on a

Table 2: β-1,3-glucanase activity in the plasma, plasma HBP and effluent

	β-1,3-glucanase activity OD/min/mg	
Samples	Control	Tested
Plasma[a]	3.0130±0.003	3.95470±0.016
Plasma HBP[b]	4.2510±0.050	4.29300±0.177
Effluent[c]	11.7494±0.056	13.12661±0.365

[a, c]Tested value significantly different from control value ($p<0.05$); [b]different between tested value and control value not significant ($p>0.05$)

Table 3: Plasma HBP identification based on region, molecular weight and isoelectric point

Identification	Molecular weight (kDa)	Isoelectric point
HBP-alpha3	38	4.6
HBP-alpha1	37	4.9
HBP-alpha2	37	5.0
HBP-beta1	37	5.3
HBP-beta2	37	5.5

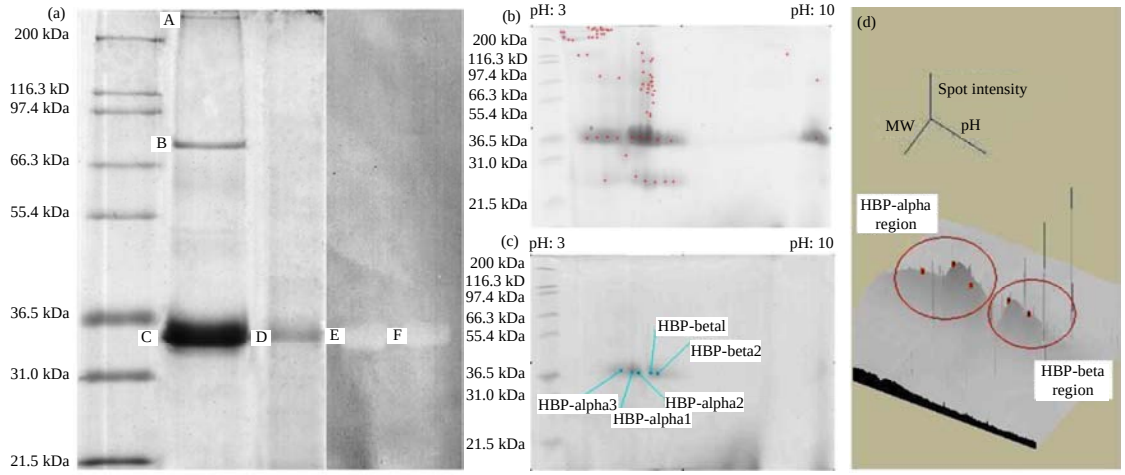

Fig. 3: SDS-PAGE analysis of the *C. iredalei* whole plasma produced three bands (a, b and c) with molecular mass estimations of >200, 72 and 35 kDa, respectively. *C. iredalei* plasma HBP appeared as a single band (d) with an estimated molecular mass of 35 kDa. Immunoblotting indicated that the HBP antiserum detected both the *C. iredalei* plasma (e) and *C. iredalei* plasma HBP (f) as a single band with an estimated molecular mass of 35 kDa; a) 2D-electrophoresis of *C. iredalei* plasma produced 66 spots as detected using the Melanie Viewer Software; b) 2D-electrophoresis of *C. iredalei* plasma HBP produced 5 spots as detected using the Melanie viewer Software; c) 3D view of the *C. iredalei* plasma HBP spots based on colour intensity, molecular weight and isoelectric point. The 3D view indicated 5 peaks, marked by red marker. The first region contains 3 peaks, named the HBP-alpha region. The second region, containing 2 peaks was named the HBP-beta region

positive charge value or isoelectric focusing Point (PI). NaCl is the best chemical to elute plasma HBP because NaCl helps in neutralising the electrostatic forces between the heparin and protein. Certain proteins with specific PIs can be eluted at certain NaCl concentrations. The study has succeeded in separating a plasma HBP that binds through electrostatic forces using a multiple step gradient that resembles ion exchange chromatography.

In the present study, an effluent containing the eluted plasma HBP from *C. iredalei* enhanced the PO activity compared to the effluent alone with β-1,3-glucan. Moreover, an increased amount of eluted plasma HBP in the effluent indicates a positive response in the PO activity. A PO activity has been discovered in several bivalve species (Asokan *et al*., 1997; Hellio *et al*., 2007; Munoz *et al*., 2006; Newton *et al*., 2004; Smith and Soderhall, 1991). Despite being activated by lipopolysaccharides and β-1,3-glucan (Soderhall and Cerenius, 1998), this system is also induced by detergents (SDS and Triton X-100) and exogenous proteases (trypsin and α-chymotrypsin) in certain species (Aladaileh *et al*., 2007; Asokan *et al*., 1997; Hellio *et al*., 2007). However, no study on the enhancement of PO activity by heparin has currently been performed. Jayaraj *et al*. (2008) tried to further characterise the bivalve PO system by focusing on BGBP. The BGBP activation of the crustacean proPO system has been well characterised and documented (Cerenius *et al*., 1994; Duvic and Soderhall, 1990; Hernandez-Lopez *et al*., 1996; Lee *et al*., 2000; Perazzolo and Barracco, 1998; Sritunyalucksana *et al*., 2002; Vargas-Albores *et al*., 1997; Yepiz-Plascencia *et al*., 1998). This present study presents evidence for PO activation in *C. iredalei* through a serine protease. Moreover, eluted plasma HBP from *C. iredalei* existence of a serine protease enzyme which is also a prophenoloxidase-activating enzyme (ppA) and its function in mediating PO activity have been studied in other invertebrate phyla but not in bivalves (Cerenius and Soderhall, 2004). The present study assumes that when eluted plasma HBP binds to β-1,3-glucan, ppA is activated from its zymogen pro-form (that is the prophenoloxidase-activating enzyme pro-ppA) through a serine protease process. Thus, this active ppA will activate other proteases in a cascade and eventually activate proPO to PO. The study indicates the presence of a β-1,3-glucanase activity in *C. iredalei* plasma. β-1,3-glucanase is an enzyme that binds to and degrades β-1,3-glucan and has been studied in other bivalves for example, *Mactra coralline*, *Tapes decussatus*, *Mytilus galloprovincialis*, *Ostrea edulis*, *Perna viridis* and *Crassostrea gigas* (Gianfreda *et al*., 1979; Itoh *et al*., 2010; Jayaraj *et al*., 2008). However, the eluted plasma HBP from *C. iredalei* did not possess a β-1,3-glucanase activity.

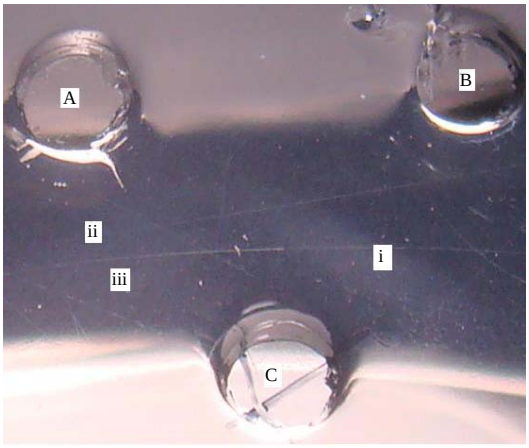

Fig. 4: Immunodiffusion test with well A contains the effluent, well B contains the plasma HBP and well C contains the antiserum against HBP. The antiserum produced a single precipitin (i) towards the plasma HBP while producing two precipitins (ii and iii) towards the effluent. The antiserum displayed a partial coalescence pattern towards the plasma HBP

Isolated eluted plasma HBP from *C. iredalei* displayed different molecular weights together with the BGBPs from other bivalves. The BGBP isolated from *P. viridis* produced 9 bands with a total molecular weight of 510 kDa while the 2 BGBPs identified from *C. gigas* displayed a single band each with molecular weights of 65.6 and 51.3 kDa, respectively (Itoh *et al*., 2010; Jayaraj *et al*., 2008). This is the first report on the 2D electrophoresis of plasma HBP based on molecular weight and PI separation. When separated by molecular weight and PI, an analysis of the 3D structure indicated that the eluted plasma HBP produced 2 major regions which were termed the α and β regions. The approximate molecular weight and PI for the α region were 37.3 kDa and 4.83 while for the β region, these values were 37 kDa and 5.4.

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Cg-EcSOD        MNALIVLSLAALAGFVSATARNEANVNIYLHLSDDEDSNYENSMHYAQCEMEPNHFMPGN 60
Cavortin        MNALIVLGLAALAGFVSATARNEANVNIYLHLSDDEDSNYENSMHYVQCEMEPNHFMPGN 60
C.i-BGBP        ------------------TARNEANV---------------------------------- 8
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Fig. 5: Amino acid aligned sequence of *C. iredalei* HBP (C.i-HBP), Cavortin from *C. gigas* (AAT44352) and extracellular superoxide dismutase (Cg-EcSOD) (AAY60161). *Conserved residue

The present study also indicates that the eluted plasma HBP from *C. iredalei* might be an isoform protein. However, there is a possibility that each form has its own specific function and research complimentarily. Itoh *et al.* (2010) determined that in addition to the occurrence of multiple BGBP that are present in *C. gigas*, their functions are different from one another. In this study, it is possible that in the eluted plasma HBP, the isoform proteins may not only play some part in activating the proPO system but also in other aspects of the *C. iredalei* immune response.

CONCLUSION

Although, the function of this isolated protein is currently unknown, its biochemical properties are similar to those of a β-1, 3-glucan-binding protein, suggesting that it may have a role in the immunoresponse of this species.

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